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# (54) Title: RECOMBINANT HUMAN ENDOTHELIAL CELL GROWTH FACTOR

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                                                                   EcoRI                          -1
                                                                     GAATTCGGGAACGCGCCACAAGCAGCAGCTGCTGAGCC
  Beta ECGF                           Acidic FGF       Alpha ECGF
  begins here                         begins here      begins here
1                                                                                                120
ATGGCTGAAGGGGAAATCACCACCTTCACAGCCCTGACCGAGAAGTTTAATCTGCCTCCAGGGAATTACAAGAAGCCCAAACTCCTCTACTGTAGCAACGGGGGCCACTTCCTGAGGATC
M  A  E  G  E  I  T  T  F  T  A  L  T  E  K  F  N  L  P  P  G  N  Y  K  K  P  K  L  L  Y  C  S  N  G  G  H  F  L  R  I

                                               PstI
CTTCCGGATGGCACAGTGGATGGGACAAGGGACAGGAGCGACCAGCACATTCAGCTGCAGCTCAGTGCGGAAAGCGTGGGGGAGGTGTATATAAAGAGTACCGAGACTGGCCAGTACTTG
L  P  D  G  T  V  D  G  T  R  D  R  S  D  Q  H  I  Q  L  Q  L  S  A  E  S  V  G  E  V  Y  I  K  S  T  E  T  G  Q  Y  L

                                                                                                 SphI    360
GCCATGGACACCGACGGGCTTTTATACGGCTCACAGACACCAAATGAGGAATGTTTGTTCCTGGAAAGGCTGGAGGAGAACCATTACAACACCTATATATCCAAGAAGCATGCAGAGAAG
A  M  D  T  D  G  L  L  Y  G  S  Q  T  P  N  E  E  C  L  F  L  E  R  L  E  E  N  H  Y  N  T  Y  I  S  K  K  H  A  E  K

                                                                                                         480
AATTGGTTTGTTGGCCTCAAGAAGAATGGGAGCTGCAAACGCGGTCCTCGGACTCACTATGGCCAGAAAGCAATCTTGTTTCTCCCCCTGCCAGTCTCTTCTGATTAAAGAGATCTGTTC
N  W  F  V  G  L  K  K  N  G  S  C  K  R  G  P  R  T  H  Y  G  Q  K  A  I  L  F  L  P  L  P  V  S  S  D  trm

                                                                                                         600
TGGTGTTGACCACTCCAGAGAAGTTTCGAGGGGTCCTCACCTGGTTGACCCCAAAAATGTTCCCTTGACCATTGGCTGCGCTAACCCCCAGCCCACAGAGCCTGAATTTGTAAGCAACTT
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## (57) Abstract

Endothelial cell growth factor is achieved through the application of recombinant DNA technology to prepare cloning vehicles encoding the ECGF protein and procedures are disclosed for recovering ECGF protein essentially free of other proteins of human origin. The product is useful for, among other purposes, diagnostic applications and as potential in the treatment of damaged blood vessels or other endothelical cell-lined structures.

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# RECOMBINANT HUMAN ENDOTHELIAL CELL GROWTH FACTOR

This invention relates to recombinant DNA-directed synthesis of certain proteins. More particularly, this invention relates to endothelial cell growth factor (ECGF), its recombinant DNA-directed synthesis, and ECGF's use in the treatment of endothelial cell damage and/or regeneration.

Endothelial cell growth factor, referred to herein as "ECGF", is a mitogen for endothelial cells _in vitro_. Growth of endothelial cells is a necessary step during the process of angiogenesis [Maciag, _Prog. Hemostasis and Thromb._, _7_:167-182 (1984); Maciag, T., Hoover, G.A., and Weinstein, R., _J. Biol. Chem._, _257_: 5333-5336 (1982)]. Bovine ECGF has been isolated by Maciag, et al., [_Science_ _225_:932-935 (1984)] using streptomycin sulfate precipitation, gel exclusion chromatography, ammonium sulfate precipitation and heparin-Sepharose affinity chromatography. Bovine ECGF purified in this manner yields a single-chain polypeptide which possesses an anionic isoelectric point and an apparent molecular weight of 20,000 [Maciag, _supra_; Schreiber, et al., _J. Cell Biol._, 101:1623-1626 (1985);

and Schreiber, et al., Proc. Natl. Acad. Sci., 82:6138-6142 (1985)]. More recently, multiple forms of bovine ECGF have been isolated by Burgess, et al., [J. Biol. Chem. 260:11389-11392 (1985)] by sodium chloride gradient elution of bovine ECGF from the heparin-Sepharose column or by reversed-phase high pressure liquid chromatography (HPLC). The two isolated polypeptides, designated alpha- and beta-ECGF have apparent molecular weights of 17,000 and 20,000, respectively. Using this procedure, the bovine ECGF contained in 8,500 ml of bovine brain extract ($6.25 \times 10^7$ total units) is concentrated into a total of 6 ml of alpha-ECGF ($3.0 \times 10^6$ units) and 3 ml of beta-ECGF ($5.2 \times 10^5$ units). This is a 9,300-fold purification of alpha-ECGF and 16,300-fold purification of beta-ECGF (Burgess, supra). Recently, murine monoclonal antibodies against bovine ECGF have been produced (Maciag, et al., supra) which may be useful in purifying bovine ECGF in a manner similar to the monoclonal antibody purification of Factor VIIIC described by Zimmerman and Fulcher in U.S. Patent No. 4,361,509.

In general, recombinant DNA techniques are known. See Methods In Enzymology. (Academic Press, New York) volumes 65 and 68 (1979); 100 and 101 (1983) and the references cited therein, all of which are incorporated herein by reference. An extensive technical discussion embodying most commonly used recombinant DNA methodologies can be found in Maniatis, et al., Molecular Cloning, Cold Spring Harbor Laboratory (1982). Genes coding for various polypeptides may be cloned by incorporating a DNA fragment coding for the polypeptide in a recombinant DNA vehicle, e.g., bacterial or viral vectors, and transforming a

suitable host. This host is typically an Escherichia coli (E. coli) strain, however, depending upon the desired product, eukaryotic hosts may be utilized. Clones incorporating the recombinant vectors are isolated and may be grown and used to produce the desired polypeptide on a large scale.

Several groups of workers have isolated mixtures of messenger RNA (mRNA) from eukaryotic cells and employed a series of enzymatic reactions to synthesize double-stranded DNA copies which are complementary to this mRNA mixture. In the first reaction, mRNA is transcribed into a single-stranded complementary DNA (cDNA) by an RNA-directed DNA poly-merase, also called reverse transcriptase. Reverse transcriptase synthesizes DNA in the 5'-3' direction, utilizes deoxyribonucleoside 5'-triphosphates as precursors, and requires both a template and a primer strand, the latter of which must have a free 3'-hydroxyl terminus. Reverse transcriptase products, whether partial or complete copies of the mRNA template, often possess short, partially double-stranded hairpins ("loops") at their 3' termini. In the second reaction, these "hairpin loops" can be exploited as primers for DNA polymerases. Preformed DNA is required both as a template and as a primer in the action of DNA polymerase. The DNA polymerase requires the presence of a DNA strand having a free 3'-hydroxyl group, to which new nucleotides are added to extend the chain in the 5'-3' direction. The products of such sequential reverse transcriptase and DNA polymerase reactions still possess a loop at one end. The apex of the loop or "fold-point" of the double-stranded DNA, which has thus been created, is substantially a single-strand segment. In the third

reaction, this single-strand segment is cleaved with the single-strand specific nuclease S1 to generate a "blunt-end" duplex DNA segment. This general method is applicable to any mRNA mixture, and is described by Buell, et al., J. Biol. Chem., 253:2483 (1978).

The resulting double-stranded cDNA mixture (ds-cDNA) is inserted into cloning vehicles by any one of many known techniques, depending at least in part on the particular vehicle used. Various insertion methods are discussed in considerable detail in Methods In Enzymology, 68:16-18 (1980), and the references cited therein.

Once the DNA segments are inserted, the cloning vehicle is used to transform a suitable host. These cloning vehicles usually impart an antibiotic resistance trait on the host. Such hosts are generally prokaryotic cells. At this point, only a few of the transformed or transfected hosts contain the desired cDNA. The sum of all transformed or transfected hosts constitutes a gene "library". The overall ds-cDNA library created by this method provides a representative sample of the coding information present in the mRNA mixture used as the starting material.

If an appropriate oligonucleotide sequence is available, it can be used to identify clones of interest in the following manner. Individual transformed or transfected cells are grown as colonies on a nitrocellulose filter paper. These colonies are lysed; the DNA released is bound tightly to the filter paper by heating. The filter paper is then incubated with a labeled oligonucleotide probe which is complementary to the structural gene of interest. The probe hybridizes with the cDNA for which it is complementary, and is identified by autoradio-

graphy. The corresponding clones are characterized in order to identify one or a combination of clones which contain all of the structural information for the desired protein. The nucleic acid sequence coding for the protein of interest is isolated and reinserted into an expression vector. The expression vector brings the cloned gene under the regulatory control of specific prokaryotic or eukaryotic control elements which allow the efficient expression (transcription and translation) of the ds-cDNA. Thus, this general technique is only applicable to those proteins for which at least a portion of their amino acid or DNA sequence is known for which an oligonucleotide probe is available. See, generally, Maniatis, et al., supra.

More recently, methods have been developed to identify specific clones by probing bacterial colonies or phage plaques with antibodies specific for the encoded protein of interest. This method can only be used with "expression vector" cloning vehicles since elaboration of the protein product is required. The structural gene is inserted into the vector adjacent to regulatory gene sequences that control expression of the protein. The cells are lysed, either by chemical methods or by a function supplied by the host cell or vector, and the protein is detected by a specific antibody and a detection system such as enzyme immunoassay. An example of this is the lambda $gt_{11}$ system described by Young and Davis, Proc. Nat'l. Acad. Sci. USA, 80:1194-1198 (1983) and Young and Davis, Science, 222:778 (1983).

The present invention has made it possible to provide readily available, large quantities of ECGF or ECGF fragments. This has been achieved with oligonucleotides whose design was based upon knowledge of the amino acid sequence of bovine ECGF and which react specifically with the ECGF cDNA. Production of ECGF is achieved through the application of recombinant DNA technology to prepare cloning vehicles encoding the ECGF protein and procedures for recovering ECGF protein essentially free of other proteins of human origin.

Accordingly, the present invention provides ECGF or its fragments essentially free of other proteins of human origin. ECGF is produced by recombinant DNA techniques in host cells or other self-replicating systems and is provided in essentially pure form. The invention further provides replicable expression vectors incorporating a DNA sequence encoding ECGF and a self-replicating host cell system transformed or transfected thereby. The host system is usually of prokaryotic, e.g., _E. coli_ or _B. subtilis_, or eukaryotic cells.

The ECGF is produced by a process which comprises (a) preparing a replicable expression vector capable of expressing the DNA sequence encoding ECGF in a suitable host cell system; (b) transforming said host system to obtain a recombinant host system; (c) maintaining said recombinant host system under conditions permitting expression of said ECGF-encoding DNA sequence to produce ECGF protein; and (d) recovering said ECGF protein. Preferably, the ECGF-encoding replicable expression vector is made by preparing a ds-cDNA preparation representative

of ECGF mRNA and incorporating the ds-cDNA into replicable expression vectors. The preferred mode of recovering ECGF comprises reacting the proteins expressed by the recombinant host system with a reagent composition comprising at least one binding step specific for ECGF. ECGF may be used as a therapeutic agent in the treatment of damaged or in regenerating blood vessels and other endothelial cell-lined structures.

## Brief Description of the Drawings

Figure 1 illustrates a general procedure for enzymatic reactions to produce cDNA clones.

Figure 2 illustrates the production of a library containing DNA fragments inserted into lambda $gt_{11}$.

Figure 3 illustrates a partial amino acid sequence of bovine alpha and beta ECGF.

Line a: Amino-terminal amino acid sequence of bovine alpha ECGF.

Line b: Amino-terminal amino acid sequence of bovine beta ECGF. The portion in parenthesis corresponds to $NH_2$-terminal segment for which sequence could not be determined; amino acid composition is shown instead. The sequence beginning with PheAsnLeu... was determined from trypsin-cleaved bovine beta ECGF.

Line c: Amino acid sequence of cyanogen bromide-cleaved bovine alpha ECGF.

Line d: Amino acid sequence of cyanogen bromide-cleaved bovine beta ECGF.

Figure 4 illustrates hydrogen-bonded base pairs.

Figure 5 illustrates the design of an oligonucleotide probe for human Endothelial Cell Growth Factor.

Figure 6 illustrates a schematic diagram of human ECGF cDNA clones 1 and 29. The open reading box represents the open reading frame encoding human beta ECGF. The EcoRI sites correspond to synthetic oligonucleotide linkers used in the construction of the cDNA library. The poly (A) tail at the 3' end of clone 1 is shown by $A_{17}$.

Figure 7 illustrates homology between human ECGF cDNA sequence and oligonucleotide probes.

Line a: Bovine trypsin- or cyanogen bromide-cleaved beta ECGF amino acid sequence.

Line b: Unique oligonucleotide probe.

Line c: Human ECGF cDNA sequence (determined from lambda ECGF clones 1 and 29).

Line d: Human ECGF amino acid sequence, deduced from cDNA sequence analysis.

Figure 8 illustrates the complete cDNA sequence of human ECGF. The cDNA inserts from ECGF clones 1 and 29 were subcloned into M13mp18 and the ECGF-encoding open reading frame and flanking regions sequenced by the chain termination method. In frame stop codons at the 5' and 3'

ends of the ECGF-encoding open reading frame are indicated by the underlined sequence and trm, respectively. The single-letter notation for amino acids is used: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

Figure 9 illustrates Northern blot analysis of ECGF mRNA. RNA was denatured in 2.2 M formaldehyde and 50% formamide and fractionated by electrophoresis in a 1.25% agarose gel containing 2.2 M formaldehyde. This was transferred to GeneScreen Plus (New England Nuclear) by blotting with 10X SSPE. Blots were hybridized to $^{32}$P-labeled nick-translated probes of ECGF clone 1 at 65°C for 16 hours in a mixture containing 2X SSPE, 20X Denhardt's solution, yeast transfer RNA (200 ug/ml), and 0.2% SDS. The membrane was subsequently washed at 65°C, twice with 2X SSPE and 0.2% SDS, then twice with 0.2X SSPE and 0.2% SDS, air-dried, and exposed overnight to Kodak XAR film with an intensifying screen. The migration of 28S and 18S RNA is noted.

Lane 1: 10 ug human brain poly(A)-containing RNA.

Lane 2: 10 ug human adult liver poly(A)-containing RNA.

As used herein, "ECGF" denotes endothelial cell growth factor or its fragments produced by cell or cell-free culture systems, in bioactive forms having the capacity to influence cellular growth, differentiation, and migration in vitro as does ECGF native to the human angiogenic process.

Different alleles of ECGF may exist in nature. These variations may be characterized by differences in the nucleotide sequence of the structural gene coding for proteins of identical biological function. It is possible to produce analogs having single or multiple amino acid substitutions, deletions, additions, or replacements. All such allelic variations, modifications, and analogs resulting in derivatives of ECGF which retain the biologically active properties of native ECGF are included within the scope of this invention.

"Expression vectors" refer to vectors which are capable of transcribing and translating DNA sequences contained therein, where such sequences are linked to other regulatory sequences capable of affecting their expression. These expression vectors must be replicable in the host organisms or systems either as episomes, bacteriophage, or as an integral part of the chromosomal DNA. One form of expression vector which is particularly suitable for use in the invention is the bacteriophage, viruses which normally inhabit and replicate in bacteria. Particularly desirable phage for this purpose are the lambda $gt_{10}$ and $gt_{11}$ phage described by Young and Davis, supra. Lambda $gt_{11}$ is a general recombinant DNA

expression vector capable of producing polypeptides specified by the inserted DNA.

To minimize degradation, upon induction with a synthetic analogue of lactose (IPTG), foreign proteins or portions thereof are synthesized fused to the prokaryotic protein B-galactosidase. The use of host cells defective in protein degradation pathways may also increase the lifetime of novel proteins produced from the induced lambda $gt_{11}$ clones. Proper expression of foreign DNA in lambda $gt_{11}$ clones will depend upon the proper orientation and reading frame of the inserted DNA with respect to the B-galactosidase promoter and translation initiating codon.

Another form of expression vector useful in recombinant DNA techniques is the plasmid - a circular unintegrated (extra-chromosomal), double-stranded DNA loop. Any other form of expression vector which serves an equivalent function is suitable for use in the process of this invention.

Recombinant vectors and methodology disclosed herein are suitable for use in host cells covering a wide range of prokaryotic and eukaryotic organisms. Prokaryotic cells are preferred for the cloning of DNA sequences and in the construction of vectors. For example, E. coli K12 strain HB101 (ATCC No. 33694), is particularly useful. Of course, other microbial strains may be used. Vectors containing replication and control sequences which are derived from species compatible with the host cell or system are used in connection with these hosts. The vector ordinarily carries an origin of replication, as well as characteristics capable of providing phenotypic selection in transformed cells. For example,

E. coli can be transformed using the vector pBR322, which contains genes for ampicillin and tetracycline resistance [Bolivar, et al., Gene, 2:95 (1977)].

These antibiotic resistance genes provide a means of identifying transformed cells. The expression vector may also contain control elements which can be used for the expression of the gene of interest. Common prokaryotic control elements used for expression of foreign DNA sequences in E. coli include the promoters and regulatory sequences derived from the B-galactosidase and tryptophan (trp) operons of E. coli, as well as the pR and pL promoters of bacteriophage lambda. Combinations of these elements have also been used (e.g., TAC, which is a fusion of the trp promoter with the lactose operator). Other promoters have also been discovered and utilized, and details concerning their nucleotide sequences have been published enabling a skilled worker to combine and exploit them functionally.

In addition to prokaryotes, eukaryotic microbes, such as yeast cultures, may also be used. Saccharomyces cerevisiae, or common baker's yeast, is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. Yeast promoters suitable for the expression of foreign DNA sequences in yeast include the promoters for 3-phosphoglycerate kinase or other glycolytic enzymes. Suitable expression vectors may contain termination signals which provide for the polyadenylation and termination of the mRNA transcript of the cloned gene. Any vector containing a yeast-compatible promoter, origin of replication, and appropriate termination sequence is suitable for expression of ECGF.

Cell lines derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from a vertebrate or invertebrate source. However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years. Examples of such useful hosts are the VERO, HeLa, mouse C127, Chinese hamster ovary (CHO), WI38, BHK, COS-7, and MDCK cell lines. Expression vectors for such cells ordinarily include an origin of replication, a promoter located in front of the gene to be expressed, RNA splice sites (if necessary), and transcriptional termination sequences.

For use in mammalian cells, the control functions (promoters and enhancers) on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently, Simian Virus 40 (SV40). Eukaryotic promoters, such as the promoter of the murine metallothionein gene [Paulakis and Hamer, Proc. Natl. Acad. Sci. 80:397-401 (1983)], may also be used. Further, it is also possible, and often desirable, to utilize the promoter or control sequences which are naturally associated with the desired gene sequence, provided such control sequences are compatible with the host system. To increase the rate of transcription, eukaryotic enhancer sequences can also be added to the construction. These sequences can be obtained from a variety of animal cells or oncogenic retroviruses such as the mouse sarcoma virus.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as that provided by SV40 or other viral

sources, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

Host cells can prepare ECGF which can be of a variety of chemical compositions. The protein is produced having methionine as its first amino acid. This methionine is present by virtue of the ATG start codon naturally existing at the origin of the structural gene or by being engineered before a segment of the structural gene. The protein may also be intracellularly or extracellularly cleaved, giving rise to the amino acid which is found naturally at the amino terminus of the protein. The protein may be produced together with either its own or a heterologous signal peptide, the signal polypeptide being specifically cleavable in an intra- or extracellular environment. Finally, ECGF may be produced by direct expression in mature form without the necessity of cleaving away any extraneous polypeptide.

Recombinant host cells refer to cells which have been transformed with vectors constructed using recombinant DNA techniques. As defined herein, ECGF is produced as a consequence of this transformation. ECGF or its fragments produced by such cells are referred to as "recombinant ECGF".

The procedures below are but some of a wide variety of well established procedures to produce specific reagents useful in the process of this invention. The general procedure for obtaining an mRNA mixture is to obtain a tissue sample or to culture cells producing the desired protein, and to extract the RNA by a process such

as that disclosed by Chirgwin, et al., Biochemistry, 18:5294 (1979). The mRNA is enriched by poly(A)mRNA-containing material by chromatography on oligo (dT) cellulose or poly(U) Sepharose, followed by elution of the poly(A) containing mRNA fraction.

The above poly(A) containing mRNA-enriched fraction is used to synthesize a single-strand complementary cDNA (ss-cDNA) using reverse transcriptase. As a consequence of DNA synthesis, a hairpin loop is formed at the 3' end of the DNA which will initiate second-strand DNA synthesis. Under appropriate conditions, this hairpin loop is used to effect synthesis of the ds-cDNA in the presence of DNA polymerase and deoxyribonucleotide triphosphates.

The resultant ds-cDNA is inserted into the expression vector by any one of many known techniques. In general, methods can be found in Maniatis, et al., supra, and Methods In Enzymology, Volumes 65 and 68 (1980); and 100 and 101 (1983). In general, the vector is linearized by at least one restriction endonuclease, which will produce at least two blunt or cohesive ends. The ds-cDNA is ligated with or joined into the vector insertion site.

If prokaryotic cells or other cells which contain substantial cell wall material are employed, the most common method of transformation with the expression vector is calcium chloride pretreatment as described by Cohen, R.N., et al., Proc. Nat'l. Acad. Sci. USA, 69:2110 (1972). If cells without cell wall barriers are used as host cells, transfection is carried out by the calcium phosphate precipitation method described by Graham and Van der Eb, Virology, 52:456 (1973). Other methods for introducing DNA into cells such as nuclear injection,

viral infection or protoplast fusion may be successfully used. The cells are then cultured on selective media, and proteins for which the expression vector encodes are produced.

Clones containing part or the entire cDNA for ECGF are identified with specific oligonucleotide probes deduced from a partial amino acid sequence determination of ECGF. This method of identification requires that the non-degenerate oligonucleotide probe be designed such that it specifically hybridizes to ECGF ds-cDNA. Clones containing ECGF cDNA sequences are isolated by radioactively labeling the oligonucleotide probe with $^{32}$P-ATP, hybridizing the radioactive oligonucleotide probe to the DNA of individual clones of a cDNA library containing ECGF-cDNA, and detection and isolation of the clones which hybridize by autoradiography. Such a cloning system is applicable to the lambda $gt_{11}$ system described by Young and Davis, supra.

Clones containing the entire sequence of ECGF are identified using as probe the cDNA insert of the ECGF recombinants isolated during the initial screening of the recombinant lambda $gt_{11}$ cDNA library with ECGF-specific oligonucleotides. Nucleotide sequencing techniques are used to determine the sequence of amino acids encoded by the cDNA fragments. This information may be used to determine the identity of the putative ECGF cDNA clones by comparison to the known amino acid sequence of the amino-terminus of bovine ECGF and of a peptide derived by cyanogen bromide cleavage of ECGF.

## EXAMPLE

### A. Preparation of Total RNA

Total RNA (messenger, ribosomal and transfer) was extracted from fresh two-day old human brain stem essentially as described by Chirgwin, _supra_, (1979). Cell pellets were homogenized in 5 volumes of a solution containing 4 M guanidine thiocyanate, and 25 mM Antifoam A (Sigma Chemical Co., St. Louis, Mo.). The homogenate was centrifuged at 6,000 rpm in a Sorvall GSA rotor for 15 minutes at 10°C. The supernatant fluid was adjusted to pH 5.0 by addition of acetic acid and the RNA precipitated by 0.75 volumes of ethanol at -20°C for two hours. RNA was collected by centrifugation and dissolved in 7.5 M guanidine hydrochloride containing 2 mM sodium citrate and 5 mM dithiothreitol. Following two additional precipitations using 0.5 volumes of ethanol, the residual guanidine hydrochloride was extracted from the precipitate with absolute ethanol. RNA was dissolved in sterile water, insoluble material removed by centrifugation, and the pellets were re-extracted with water. The RNA was adjusted to 0.2 M potassium acetate and precipitated by addition of 2.5 volumes of ethanol at -20°C overnight.

### B. Preparation of Poly(A)-containing RNA

The total RNA precipitate, prepared as described above, was dissolved in 20 mM Hepes buffer (pH 7.2) containing 10 mM EDTA and 1% SDS, heated at 65°C for 10 minutes, then quickly cooled to 25°C. The RNA solution was then diluted with an equal volume of water, and NaCl was added to bring the final concentration to 300 mM NaCl. Samples containing up to 240 $A_{260}$ units of RNA

were chromotagraphed on poly(U)-Sepharose using standard procedures. Poly(A)-containing RNA was eluted with 70% formamide containing 1 mM Hepes buffer (pH 7.2), and 2 mM EDTA. The eluate was adjusted to 0.24 M NaCl and the RNA was precipitated by 2.5 volumes of ethanol at -20°C.

C. Construction of cDNA Clones in Lambda $gt_{11}$

The procedure followed for the enzymatic reaction is shown in Figure 1. The mRNA (20μg) was copied into ds-cDNA with reverse transcriptase and DNA polymerase I exactly as described by Buell, et al., supra, and Wilkensen, et al., J. Biol. Chem., 253:2483 (1978). The ds-cDNA was desalted on Sephadex G-50 and the void-volume fractions further purified on an Elutip-D column (Schleicher & Schuell, Keene, NH) following the manufacturer's directions. The ds-cDNA was made blunt-ended by incubation with S1 nuclease [Ricca, et al., J. Biol. Chem., 256:10362 (1981)]. The reaction mixture consisted of 0.2 M sodium acetate (pH 4.5), 0.4 M sodium chloride, 2.5 mM zinc acetate and 0.1 unit of S1 nuclease per ng of ds-cDNA, made to a final reaction volume of 100μl. The ds-cDNA was incubated at 37°C for one hour, extracted with phenol:chloroform, and then desalted on a Sephadex G-50 column as described above.

The ds-cDNA was then treated with EcoRI methylase and Klenow fragment of DNA polymerase I using reaction conditions described in Maniatis, et al., Molecular Cloning, supra. The cDNA was again desalted on Sephadex G-50 as described above and then ligated to 0.5μg of phosphorylated EcoRI linkers using $T_4$ DNA ligase (Maniatis, et al., supra). The mixture was cleaved with EcoRI and fractionated on an 8% acrylamide gel in Tris-

borate buffer (Maniatis, et al., *supra*). DNA with a size greater than 1 kilobase was eluted from the gel and recovered by binding to an Elutip-D column, eluted with 1 M NaCl and then collected by ethanol precipitation.

As shown in Figure 2, the DNA fragments were then inserted into EcoRI cleaved and phosphatase-treated lambda $gt_{11}$, using $T_4$ DNA ligase. A library of $5.7 \times 10^6$ phage was produced, of which approximately 65% were recombinant phage. The library was amplified by producing plate stocks at 42°C on *E. coli* Y1088 [supE supF metB trpR $hsdR^-$ $hsdM^+$ tonA21 strA lacU169 (proC::Tn5) (pMC9)]. Amplification procedures are described in Maniatis, et al., *supra*. Important features of this strain, described by Young and Davis, *supra*, include (1) supF (required suppression of the phage amber mutation in the S gene), (2) $hsdR^-$ $hsdM^+$ (necessary to prevent restriction of foreign DNA prior to host modification), and (3) lacU169 (proC::Tn5), and (4) (pMC9) (a lac I-bearing pBR322 derivative which represses, in the absence of an inducer, the expression of foreign genes that may be detrimental to phage and/or cell growth).

D. Identification of Clones Containing ECGF Sequence

To screen the library for recombinant phage containing ECGF cDNA, $1.5 \times 10^6$ phage were plated on a lawn of *E. coli* Y1090 [$\Delta$lacU169 proA$\Delta$ lon araD139 strA supF (trpC22::Tn10) (pMC9)] and incubated at 42°C for 6 hours. After the plates were refrigerated overnight, a nitrocellulose filter was overlaid on the plates. The position of the filter was marked with a needle. The filter removed after one minute and left to dry at room temperature. From each plate, a duplicate filter was

prepared exactly as described, except that the filter was left in contact with the plate for 5 minutes. All filters were then prepared for hybridization, as described in Maniatis, et al., supra. This involved DNA denaturation in 0.5 M NaOH, 1.5 M NaCl, neutralization in 1 M Tris-HCl, pH 7.5, 1.5 M NaCl, and heating of the filters for 2 hours at 80°C in vacuo.

To screen the human brain stem cDNA library for clones containing ECGF inserts, a specific oligonucleotide was designed. This oligonucleotide was based upon a partial amino acid sequence analysis of the amino terminus of ECGF. As shown in Figure 3, lines a & b, bovine ECGF is isolated as two species, designated alpha and beta ECGF, which differ only in the amino acids found at the respective amino termini. As shown in Figure 3, line b, beta-ECGF is a slightly larger species than alpha-ECGF. The exact amino acid sequence at the amino terminus of beta-ECGF is undetermined, however, a sequence derived from fast atom bombardment mass spectral analysis and the amino acid composition of the amino terminal tryptic peptide of bovine beta-ECGF is shown. The amino terminal blocking group appears to be acetyl. If intact beta-ECGF is cleaved by trypsin, a second amino amino acid sequence found in beta but not alpha ACGF starting with PheAsnLeu... is determined. This sequence is also found at the amino terminus of acidic fibroblast growth factor [Thomas, K.A. et al., Prac. Natl. Acad. Sci., 82:6409-6413 (1985)]. The amino terminus of alpha-ECGF is AsnTyrLys... (Figure 3, line a) and is the equivalent of beta-ECGF minus an amino terminal extension. In Figure 3, lines c and d set forth for comparison the amino acid sequence of cyanogen bromide-cleaved bovine alpha and beta ECGF, respectively.

For oligonucleotide design, the amino acid sequence IleLeuProAspGlyThrValAspGlyThrLys, corresponding to alpha-ECGF amino acids 19-29 inclusive, was chosen. Rather then design a mixture of oligonucleotides covering all of the possible coding sequences (owing to the degeneracy of the genetic code), a long unique oligonucleotide was designed. Such oligonucleotide probes have been previously shown to be successful probes in screening complex cDNA [Jaye, et al., Nucleic Acids Research 11:2325-2335, (1983)] and genomic [Gitschier, et al., Nature, 312:326-330 (1984)] libraries. Three criteria were used in designing the ECGF probe: (1) The dinucleotide CG was avoided. This strategy was based upon the observed underrepresentation of the CG dinucleotide in eukaryotic DNA [Josse, et al., J. Biol. Chem. 236:864-875, (1961)]; (2) preferred codon utilization data was used wherever possible. A recent and comprehensive analysis of human codon utilization was found in Lathe, J. Mol. Biol. 183:1-12 (1985); and (3) wherever the strategies of CG dinucleotide and preferred codon utilization were uninformative, unusual base pairing was allowed. This strategy was based upon the natural occurence of G:T, I:T, I:A and I:C base pairs which occur in the interaction between tRNA anticodons and mRNA codons [Crick, J. Mol. Biol. 19:548-555, (1966)]. A diagram of usual and unusual base pairs is shown in Figure 4. Use of I (Inosine) in a hybridization probe was first demonstrated, in a model experiment, by Ohtsuka, et al., J. Biol. Chem. 260:2605-2608 (1985). The overall strategy and choice made in the design of the oligonucleotide used to screen the human brain stem cDNA library for ECGF is shown in Figure 5. In addition, two other oligonucleotides, designed with the same strategy, were constructed.

Approximately 30 pmole of the oligonucleotide shown in Figure 5 were radioactively labeled by incubation with $^{32}$P-gamma-ATP and T4 polynucleotide kinase, essentially as described by Maniatis, et al., supra. Nitrocellulose filters, prepared as described above, were prehybridized at 42°C in 6X SSPE (1X SSPE = 0.18M NaCl, 0.01M $NaHPO_4$ pH 7.2, 0.001M EDTA), 2X Denhardt's (1X Denhardt's - 0.02% each Ficoll, polyvinylpyrrolidone, bovine serum albumin), 5% dextran sulfate, and 100 $\mu$g/ml denatured salmon sperm DNA. The $^{32}$P-labeled oligonucleotide was added following four hours of prehybridization, and hybridization continued overnight at 42°C. Unhybridized probe was removed by sequential washing at 37°C in 2X SSPE, 0.1% SDS.

From 1.5 x $10^6$ plaques screened, 2 plaques gave positive autoradiographic signals after overnight exposure. These clones were purified to homogeneity by repeated cycles of purification using the above oligonucleotide as hybridization probe.

The two clones that were isolated, ECGF clones 1 and 29, were analyzed in further detail. Upon digestion with EcoRI, clone 1 and 29 revealed cDNA inserts of 2.2 and 0.3 Kb, respectively. Nick translation of cloned cDNA and its subsequent use as a radiolabeled probe in Southern blot analysis (Maniatis, et al., supra) revealed that clones 1 and 29 were related and overlapping clones. The overlapping nature of these two clones is shown in Figure 6.

Clones 1 and 29 were analyzed in further detail as follows: An additional two oligonucleotides were designed, based upon the amino acid sequence of bovine ECGF. These oligonucleotides were designed based upon the

same considerations as those used in the design of the oligonucleotide used to isolate clones 1 and 29. These oligonucleotides (ECGF oligonucleotides II and III) are shown in Figure 7. These two oligonucleotides as well as oligo(dT)$_{18}$ were radioactively labeled in a kination reaction as described above and used as hybridization probes in Southern blotting experiments. The results of these experiments showed that the 0.3 Kb cDNA insert of clone 29 hybridized to ECGF oligonucleotides I and II but not to ECGF oligonucleotide III or oligo(dT)$_{18}$; the 2.2 Kb cDNA insert of clone 1 hybridized to oligonucleotide I, II, III as well as oligo(dT)$_{18}$. These data and subsequent nucleotide sequence determination of clones 1 and 29 showed that the 3' end of clone 1 ends with a poly(A) tail. Hybridization of clone 1 to ECGF oligonucleotide III, which is based on a cyanogen bromide cleavage product of bovine ECGF, as well as to oligo(dT)$_{18}$, strongly suggested that this clone contains the rest of the coding sequence for both alpha and beta ECGFs as well as a large (greater than 1 Kb) 3' flanking sequence.

The cDNA inserts from clones 1 and 29 were isolated, subcloned into M13mp18, and the ECGF-encoding open reading frame and flanking regions sequenced by the chain termination method [Sanger et al., Proc. Natl. Acad. Sci. USA 74:5463-5467 (1977)]. The nucleotide sequence of these clones and the amino acid sequence deduced from the nucleic acid sequence is shown in Figure 8. Examination of the nucleotide sequence reveals an open reading frame of 465 nucleotides encoding human ECGF. The 155 amino acids of human ECGF were found to be flanked by translation stop codons. The $NH_2$-terminal amino acid of

human beta ECGF deduced from the cDNA sequence is methionine, which most likely serves as the translation initiation residue. These data, together with the relatively non-hydrophobic nature of the first 15-20 amino terminal residues, strongly suggest that human beta ECGF is synthesized without a $NH_2$-terminal signal peptide. A comparison of Figures 3 and 8 shows that the amino terminal amino acid sequence of trypsin-cleaved bovine beta ECGF as well as that of bovine alpha ECGF are nearly identical to the amino acid sequence predicted from the nucleotide sequence of lambda ECGF clones 1 and 29. An overall homology between the two species of over 95% is observed.

Northern blot analysis (Maniatis, et al, supra) reveals that ECGF mRNA is a single molecular species which comigrates with 28S rRNA (Figure 9). Considering the variation in the estimated size of 28S rRNA, the approximate size of ECGF mRNA is 4.8 $\pm$ 1.4 Kb. All of the sequence encoding the mature forms of both alpha and beta ECGF is encoded within ECGF clones 1 and 29, which together encompasses approximately 2.3 Kb. Thus, these data demonstrate that the region 5' and flanking the ECGF-encoding sequences, is very large (approximately 2.5 $\pm$ 1.4 Kb).

cDNA inserts from clone 1 and clone 29 were excised by digestion with EcoRI and subcloned in pUC8 at the EcoRI site. The plasmid formed from clone 1 was designated pDH15 and the plasmid formed from clone 29 was designated pDH14. The plasmids were deposited in American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852. The plasmid from clone 1, pDH15, was designated ATCC 53336 and the plasmid from clone 29, pDH 14, was designated ATCC 53335.

Thus, this example describes experimental procedures which provide human endothelial cell growth factor, essentially free of other proteins of human origin.

ECGF has utility in the growth and amplification of endothelial cells in culture. Currently, ECGF for cell culture use is extracted from bovine brain by the protocol of Maciag, et al., [Proc. Natl. Acad. Sci., 76:11, 5674-5678 (1978)]. This crude bovine ECGF is mitogenic for human umbilical vein endothelial cells [Maciag, et al., J. Biol. Chem 257:5333-5336 (1982)] and endothelial cells from other species. Utilization of heparin with ECGF and a fibronectin matrix permits the establishment of stable endothelial cell clones. The recommended concentration of this crude bovine ECGF for use as a mitogen in vitro is 150 micrograms per milliliter of growth medium.

Recombinant DNA-derived human ECGF has utility, therefore, as an improved substitute for crude bovine ECGF in the in vitro culturing of human endothelial cells and other mesenchymal cells for research use. The activity of human ECGF is expected to be the same as or better than bovine ECGF in the potentiation of endothelial cell growth due to the high degree of homology in the amino acid sequences of both proteins. The expected effective dose range for potentiating cell division and growth in vitro is 5 - 10 ng of purified ECGF per milliliter of culture medium. Production of the ECGF via recombinant-DNA technologies as outlined in this patent application and subsequent purification as described by Burgess, et al., [J. Biol. Chem. 260:11389-11392 (1985)] will provide large quantities of a pure product of human origin (heretofore

unavailable in any quantity or purity) with which to develop models of human homeostatis and angiogenesis.

Recombinant DNA-derived human ECGF also has utility in the potentiation of cell growth on a prosthetic device, rather than a tissue culture flask or bottle. This device may or may not be coated with other molecules which would facilitate the attachment of endothelial cells to the device. These facilitating molecules may include extracellular matrix proteins (eg. fibronectin, laminin, or one of the collagens), human serum albumin, heperin or other glycosaminoclycans or inert organic molecules. Endothelial cells would be cultured on these surfaces using effective doses of ECGF in the culture medium, ultimately covering the device with an endothelial cell monolayer. This device would then provide a non-thrombogenic surface on the prosthetic device, thus reducing the risk of potentially life-threatening thrombogenic events subsequent to implantation of the prosthetic device.

ECGF has utility in diagnostic applications. Schreiber, et al., [Proc. Natl. Acad. Sci. 82:6138 (1985)] developed a double antibody immunoassay for bovine ECGF. In this assay, 96-well polyvinyl chloride plates were coated with rabbit anti-ECGF and the remaining binding sites subsequently blocked with 10% normal rabbit serum. Samples of ECGF were then added to the wells and incubated. After washing, murine monoclonal anti-ECGF was added. After incubation and several washes, rabbit anti-mouse IgG coupled with peroxidase was added. The reaction product was quantitated spectrophotometrically after conversion of O-phenylenediamine in the presence of hydrogen peroxide. A similarly constructed immunoassay may be useful for monitoring human ECGF levels in disease

states affecting endothelial cell growth. Purified recombinant-DNA derived ECGF would be useful as a standard reagent in quantifying unknown ECGF samples.

ECGF also may have potential in the treatment of damaged or in the regeneration of blood vessels and other endothelial cell-lined structures.

It should be appreciated that the present invention is not to be construed as being limited by the illustrative embodiment. It is possible to produce still other embodiments without departing from the inventive concepts herein disclosed. Such embodiments are within the ability of those skilled in the art.

WHAT IS CLAIMED IS:

1. A process for producing human endothelial cell growth factor comprising, providing a replicable expression vector capable of expressing the DNA sequence encoding human endothelial cell growth factor in a suitable host, transforming said host to obtain a recombinant host, and maintaining said recombinant host under conditions permitting expression of said endothelial cell growth factor - encoding cDNA sequence to produce endothelial cell growth factor.

2. The process according to claim 1 including the further step of recovering said endothelial cell growth factor.

3. The process according to claim 2 wherein said expression vector is a bacteriophage.

4. The process according to claim 3 wherein said bacteriophage is a member of the group consisting of lambda $gt_{10}$ and lambda $gt_{11}$.

5. The process according to claim 2 wherein said expression vector is a plasmid.

6. The process according to claim 5 wherein said plasmid is derived from pBR322.

7. The process according to claim 2 wherein the control function on said expression vector is provided by viral material.

8. The process according to claim 7 wherein said viral material is a member of the group consisting of bovine papilloma virus, Epstein Barr virus, adenovirus, Simian virus 40 and bacculovirus.

9. Endothelial cell growth factor produced according to the process of claim 2.

10. A replicable expression vector capable of expressing endothelial cell growth factor in a self-replicating recombinant system.

11. The self-replicating recombinant system transformed with the vector of claim 10.

12. The recombinant system according to claim 11 wherein said system is in a cell.

13. The recombinant system according to claim 11 wherein said system is cell-free.

14. The recombinant system according to claim 11 obtained by transforming or infecting a member of the group consisting of an E. coli, B. subtilis, insect, yeast and vertebrate cell.

15. The recombinant system according to claim 11 obtained by transforming eukaryotes.

16. The process according to claim 1 wherein recovering said endothelial cell growth factor comprises reaction of the proteins expressed by the recombinant host

system with a reagent composition comprising at least one binding protein specific for endothelial cell growth factor.

17. Human endothelial cell growth factor essentially free of other proteins of human origin.

18. The endothelial cell growth factor of claim 17 comprising a polypeptide sequence extending from the $NH_2$-terminal amino acid of endothelial cell growth factor.

19. A cDNA clone comprising the complete coding sequence for human endothelial cell growth factor.

20. A cDNA clone containing untranslated nucleotide sequences located 5' and 3' to the coding sequence of human endothelial cell growth factor as defined by the endothelial cell growth factor mRNA.

21. A composition comprising a therapeutically effective amount of human endothelial cell growth factor to promote wound healing in a mixture with an acceptable carrier.

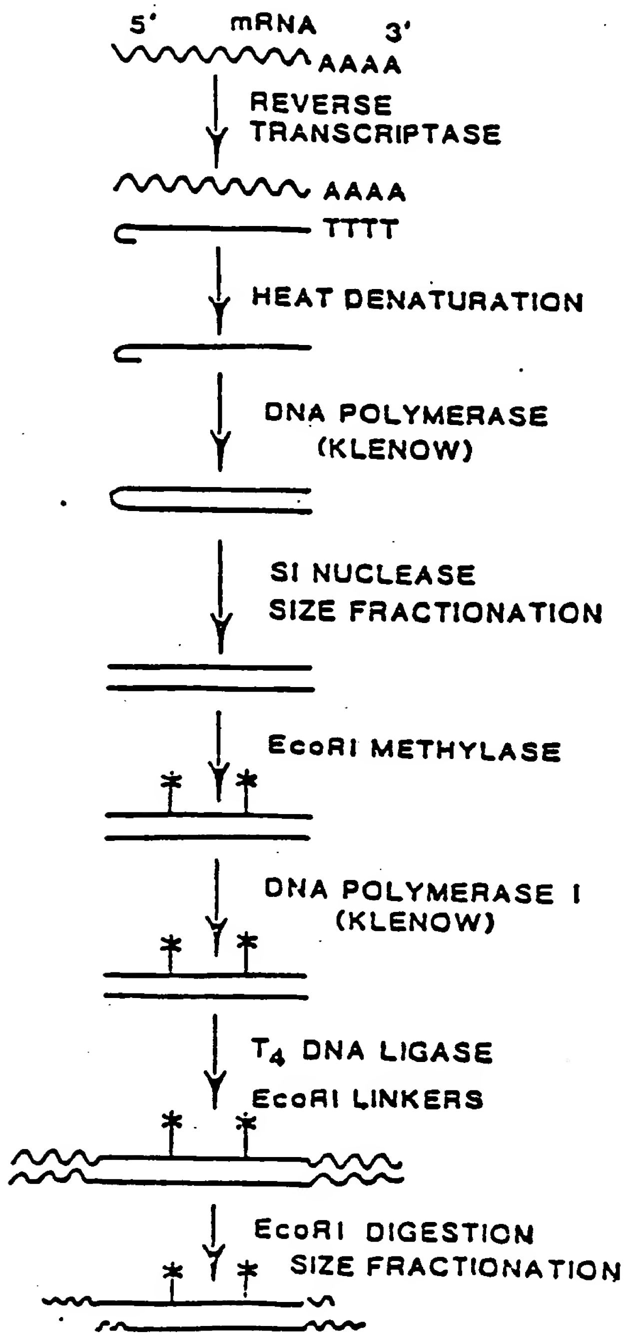
5'
mRNA
3'
AAAA
REVERSE
TRANSCRIPTASE
AAAA
TTTT
HEAT DENATURATION
DNA POLYMERASE
(KLENOW)
SI NUCLEASE
SIZE FRACTIONATION
EcoRI METHYLASE
DNA POLYMERASE I
(KLENOW)
T4 DNA LIGASE
EcoRI LINKERS
EcoRI DIGESTION
SIZE FRACTIONATION

FIG. I

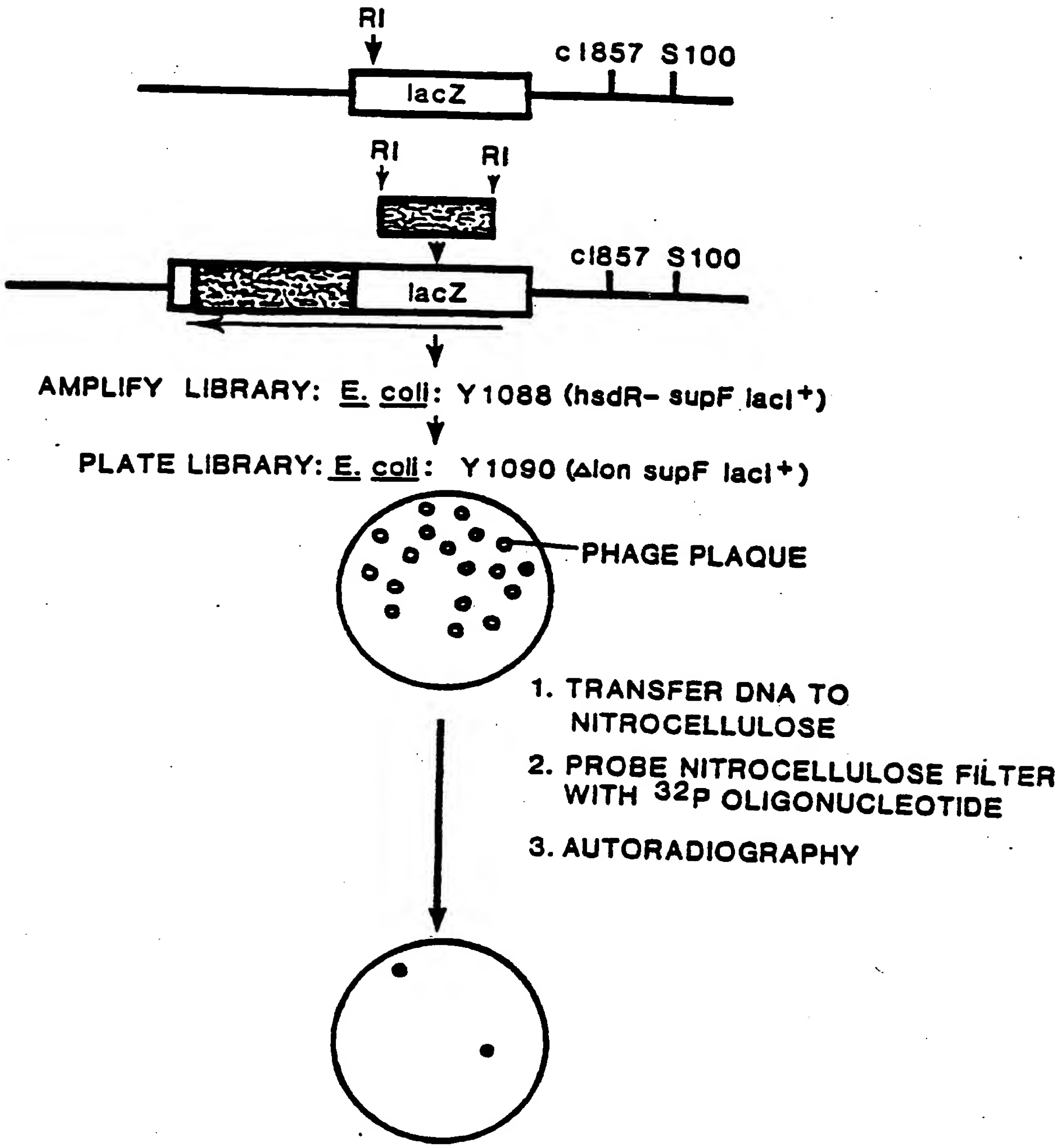
RI
c1857 S100
lacZ
RI RI
c1857 S100
lacZ
AMPLIFY LIBRARY: E. coli: Y1088 (hsdR- supF lacI+)
PLATE LIBRARY: E. coli: Y1090 (Δlon supF lacI+)
PHAGE PLAQUE
1. TRANSFER DNA TO NITROCELLULOSE
2. PROBE NITROCELLULOSE FILTER WITH 32P OLIGONUCLEOTIDE
3. AUTORADIOGRAPHY

FIG.2

5

a. $(H_2N)$-AsnTyrLysLysPro

b. AcAlaGluGlyGluThrThrThrPheThrAlaLeuThrGluLysPheAsnLeuProLeuGlyAsnTyrLysLysPro

10 15 20 25 30

a. LysLeuLeuTyrCysSerAsnGlyGlyTyrPheLeuArgIleLeuProAspGlyThrValAspGlyThrLysAspAspHis...

b. LysLeuLeuTyrCysSerAsnGlyGlyTyrPheLeuArgIleLeuProAspGlyThrValAspGlyThrLysAspAspHis...

c. AspThrAspGluLeuLeuTyrGlySerGlnThrProAsnGluGlu

d. AspThrAspGluLeuLeuTyrGlySerGlnThrProAsnGluGlu

FIG.3

## Hydrogen-Bonded Base Pairs

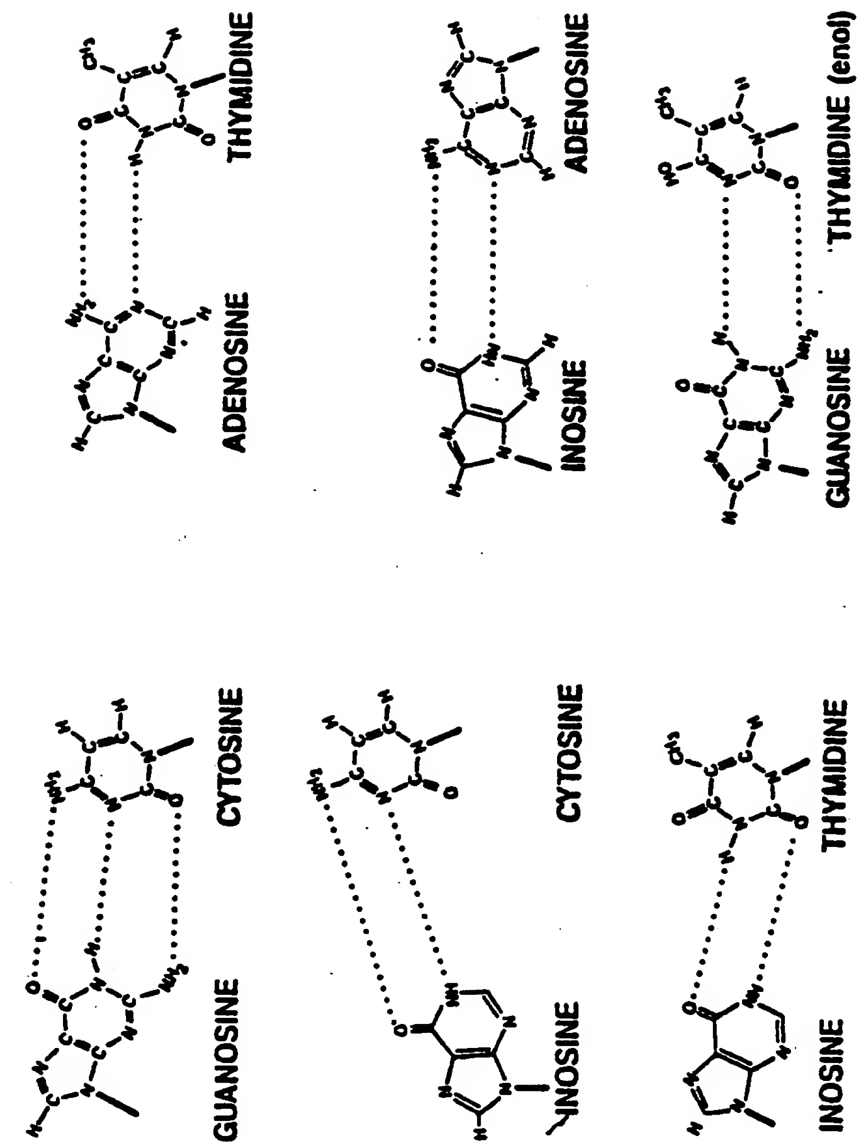


FIG.4

DESIGN OF AN OLIGONUCLEOTIDE PROBE FOR

HUMAN ENDOTHELIAL CELL GROWTH FACTOR

bovine ECGF protein sequence: I L P D G T V D G T K

degenerate coding sequence: $AT^{A}_{T}$ CTX CCX $GA^{C}_{T}$ GGX ACX GTX $GA^{C}_{T}$ GGX ACX $AA^{A}_{G}$
C $TT^{A}_{G}$

$3 \times 6 \times 4 \times 2 \times 4 \times 4 \times 4 \times 2 \times 4 \times 2 \times 2 = 2.95 \times 10^{5}$

$AT^{A}_{T}$ CTX CCX $GA^{C}_{T}$ GGX ACX GTX $GA^{C}_{T}$ GGX ACX $AA^{A}_{G}$
C $TT^{A}_{G}$

CG dinucleotide: not C or G; not C; not C or G; not C; not G

$AT^{A}_{T,C}$ $^{C}_{T}T^{C,G,A}_{T}$ $CC^{A}_{T}$ GAT GGX $AC^{A}_{T}$ GTX $GA^{C}_{T}$ GGX ACX $AA^{A}_{G}$

$3 \times 8 \times 2 \times 1 \times 4 \times 2 \times 4 \times 2 \times 4 \times 4 \times 2 = 9.84 \times 10^{4}$

codon usage: ATT $^{C}_{T}TT$ $CC^{A}_{T}$ GAT GGX $AC^{A}_{T}$ GTX GAT GGX $AC^{A}_{T,C}$ AAA

$1 \times 2 \times 2 \times 1 \times 4 \times 2 \times 4 \times 1 \times 4 \times 3 \times 1 = 1.54 \times 10^{3}$

allowance for inosine and G:T basepairs: ATT TTT CCI GAT GGI ACI GTI GAT GGI ACI AAA

16 A/T
11 G/C
6 I
33

FIG.5

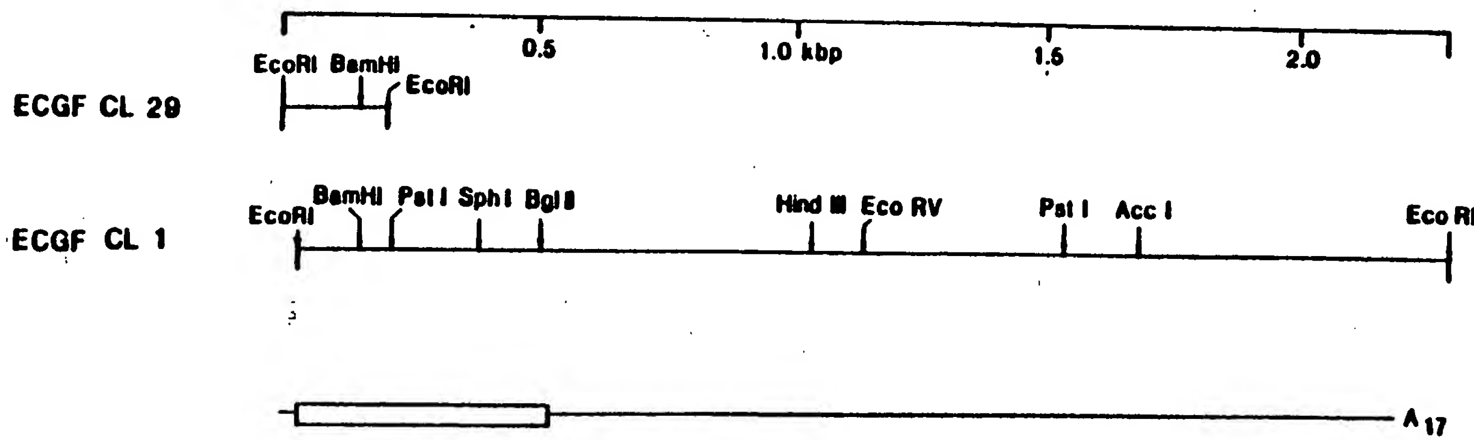
ECGF CL 29
ECGF CL 1
0.5
1.0 kbp
1.5
2.0
EcoRI BamHI
EcoRI
EcoRI
BamHI Pst I Sph I BglII
Hind III Eco RV
Pst I Acc I
Eco RI
A 17

FIG.6

HOMOLOGY BETWEEN HUMAN ECGF cDNA SEQUENCE AND OLIGONUCLEOTIDE PROBES

```
a.   F  N  L  P  L  G  N  Y  K  K  P  K  L  L  Y  C  S  N  G  G  Y  F  L  R  I  L  P  D  G  T  V  D  G  T  K
b.                    AACTACAAAAAACCIAAACTICTITACTGCTCIAACGGIGGITACTTC      ATTTTCCIGATGGIACIGTIGATGGIACIAAA
                      :: ::::: :: :: ::::: :: :::::    :::::/:: /:::::      :://::::/::::: ::/::/:::::/::/:
c.  TTTAATCTGCCTCCAGGGAATTACAAGAAGCCCAAACTCCTCTACTGTAGCAACGGGGGCCACTTCCTGAGGATCCTTCCGGATGGCACAGTGGATGGGACAAGG
d.   F  N  L  P  L  G  N  Y  K  K  P  K  L  L  Y  C  S  N  G  G  Y  F  L  R  I  L  P  D  G  T  V  D  G  T  K
                          ECGF Oligonucleotide II*                          ECGF Oligonucleotide I


a.   M  D  T  D  G  L  L  Y  G  S  Q  T  P  N  E  E
b.  ATGGACACIGACGGICTICTITACGGITCICAGACICCIAACGAGGAG
    :::::::: :::::/::/ :/::::: ::/:::::/::/::/:::::/
c.  ATGGACACCGACGGGCTTTTATACGGCTCACAGACACCAAATGAGGAA
d.   M  D  T  D  G  L  L  Y  G  S  Q  T  P  N  E  E
              ECGF Oligonucleotide III*
```

a. Bovine ECGF protein sequence

b. Oligonucleotide probe

c. Human ECGF cDNA sequence

d. Human ECGF deduced amino acid sequence

: Signifies G:C or A:T base pairing

/ Signifies unusual base pairing

* Actual oligonucleotide used was the complement of the sequence shown

FIG. 7

```
                                                                                   EcoRI                       -1
                                                                                     GAATTCGGGAACGCGCCACAAGCAGCAGCTGCTGAGCC
   Beta ECGF                                  Acidic FGF         Alpha ECGF
   begins here                                begins here        begins here
1                                                                                                              120
ATGGCTGAAGGGGAAATCACCACCTTCACAGCCCTGACCGAGAAGTTTAATCTGCCTCCAGGGAATTACAAGAAGCCCAAACTCCTCTACTGTAGCAACGGGGGCCACTTCCTGAGGATC
M [A] E G E I T.T F T A L T E K [F] N L P P G [N] Y K K P K L L Y C S N G G H F L R I

                                                                PstI
CTTCCGGATGGCACAGTGGATGGGACAAGGGACAGGAGCGACCAGCACATTCAGCTGCAGCTCAGTGCGGAAAGCGTGGGGGAGGTGTATATAAAGAGTACCGAGACTGGCCAGTACTTG
L P D G T V D G T R D R S D Q H I Q L Q L S A E S V G E V Y I K S T E T G Q Y L

                                                                                                        SphI   360
GCCATGGACACCGACGGGCTTTTATACGGCTCACAGACACCAAATGAGGAATGTTTGTTCCTGGAAAGGCTGGAGGAGAACCATTACAACACCTATATATCCAAGAAGCATGCAGAGAAG
A M D T D G L L Y G S Q T P N E E C L F L E R L E E N H Y N T Y I S K K H A E K

                                                                                                               480
AATTGGTTTGTTGGCCTCAAGAAGAATGGGAGCTGCAAACGCGGTCCTCGGACTCACTATGGCCAGAAAGCAATCTTGTTTCTCCCCCTGCCAGTCTCTTCTGATTAAAGAGATCTGTTC
N W.F V G L K K N G S C K R G P R T H Y G Q K A I L F L P L P V S S D trm

                                                                                                               600
TGGTGTTGACCACTCCAGAGAAGTTTCGAGGGGTCCTCACCTGGTTGACCCCAAAAATGTTCCCTTGACCATTGGCTGCGCTAACCCCCAGCCCACAGAGCCTGAATTTGTAAGCAACTT
```

FIG.8

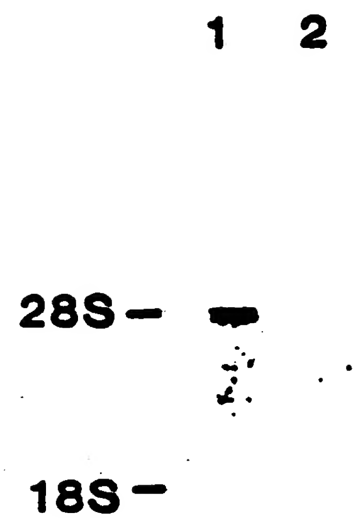


FIG.9

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/00425

**I. CLASSIFICATION OF SUBJECT MATTER** (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C12P 21/00, 21/04; C12N 15/00, 1/00, 5/00, 1/20, 1/16 C07K 7/64, 15/00; A61K 37/00

**II. FIELDS SEARCHED**

Minimum Documentation Searched [4]

| Classification System | Classification Symbols |
|---|---|
| U.S. | 435, 68, 71, 172.3, 240, 253, 255, 317; 935/13; 530/399; 424/177; 514/8, 25 |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

COMPUTER SEARCH, CAS, BIOSIS: ENDOTHELIAL CELL GROWTH FACTOR

**III. DOCUMENTS CONSIDERED TO BE RELEVANT** [14]

| Category * | Citation of Document, [16] with indication, where appropriate, of the relevant passages [17] | Relevant to Claim No. [18] |
|---|---|---|
| Y<br>X | The Journal of Biological Chemistry Vol. 257, issued May 25, 1982 (Baltimore Maryland, USA), (MACIAG ET AL), "High and Low Molecular Weight Forms of Endothelial Cell Growth Factor," pages 5333-5336. | 1-21<br>9,17,18 |
| Y<br>X | The Journal of Biological Chemistry Vol. 260, issued September 25, 1985 (Baltimore Maryland, USA), (BURGESS ET AL), "Multiple Forms of Endothelial Cell Growth Factor," pages 11389-11392. | 1-21<br>9,17,18 |
| Y<br>X | Biochemistry and Biophysical Research Communications Vol. 124, issued October 15, 1984, (New York, USA), (CONN ET AL), "The Isolation and Purification of Two Anionic Endothelial Cell Growth Factors From Human Brain," pages 262-268. | 1-21<br>9,17,18 |

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

**IV. CERTIFICATION**

| Date of the Actual Completion of the International Search [2] | Date of Mailing of this International Search Report [2] |
|---|---|
| 15 April 1987 | 30 APR 1987 |
| **International Searching Authority [1]** | **Signature of Authorized Officer [10]** |
| ISA/US | Alvin E. Tanenholtz |

Form PCT/ISA/210 (second sheet) (May 1986)

| | | |
|---|---|---|
| Y | The Journal of Biological Chemistry Vol. 256 issued August 10, 1981 (Baltimore Maryland, USA), (HEWICK ET AL), "A Gas-Liquid Solid Phase Peptide and Protein Sequenator," pages 7990-7997. | 1-21 |
| Y | J. Mol. Biol. Vol. 183 issued 1985, (London England), (LATHE), "Synthetic Oligonucleotide Probes Deduced from Amino Acid Sequence Data, Theoretical and Practical Considerations," pages 1-12. | 1-21 |

**V.☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]**

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☐ Claim numbers , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2.☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

**VI.☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]**

This International Searching Authority found multiple inventions in this international application as follows:

1.☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

International Application No. PCT/US87/00425

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, [16] with indication, where appropriate, of the relevant passages [17] | Relevant to Claim No [18] |
|---|---|---|
| Y | Proc. Natl. Acad. Sci. USA Vol. 80 issued March 1983 (Washington, D.C.), (YOUNG ET AL), "Efficient isolation of genes using antibody probes," pages 1194-1198. | 1-21 |
| Y | Nucleic Acids Research Vol. 11 issued 1983, (Oxford England), (JAYE ET AL), "Isolation of a human anti-haemophilic factor IX cDNA clone using a unique 52-base synthetic oligonucleotide probe deduced from the amino acid sequence of bovine factor IX, pages 2325-2335. | 1-21 |